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NITROGENASE FROM *RHODOSPIRILLUM RUBRUM*

RELATION BETWEEN 'SWITCH-OFF' EFFECT AND THE MEMBRANE COMPONENT. HYDROGEN PRODUCTION AND ACETYLENE REDUCTION WITH DIFFERENT NITROGENASE COMPONENT RATIOS

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Summary

Nitrogenase activity of 'membrane-free' extracts, produced from nitrogen-starved *Rhodospirillum rubrum* to which 4 mM NH_4^+ had been added is only about 10% of the activity in the control. The activity could be restored to 80% by including the membrane component, earlier found to activate *R. rubrum* nitrogenase, in the reaction mixture. The relation between this 'switch-off/switch-on' effect and the function of the membrane component is discussed.

Hydrogen production catalyzed by *R. rubrum* nitrogenase is also dependent on activation by the membrane component. Hydrogen production is inhibited by acetylene but the degree of inhibition is dependent on the nitrogenase component ratio. The strongest inhibition is achieved at low MoFe protein/Fe protein ratios. The ATP/2 e^- values are 4—5 at the component ratios giving the highest activity and increase at high MoFe protein/Fe protein ratios. CO inhibits acetylene reduction but has no effect on the hydrogen production.

Introduction

Nitrogenase has been isolated and characterized from a number of organisms, mainly non-photosynthetic. In all cases the enzyme complex consists of two components, the MoFe protein and the Fe protein, both of which are required

Abbreviation: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

in the ATP-dependent reductions of substrates [1]. The fact that nitrogenase can reduce a number of substrates, e.g. H^+, C_2H_2, N_2, has been used to investigate the mechanism of reduction [2,3].

Rhodospirillum rubrum nitrogenase has been isolated and characterized by us [4] and by Ludden and Burris [5] and found to be similar to nitrogenases from other organisms. In one functional respect though *R. rubrum* nitrogenase differs from other nitrogenases since it requires activation by a membrane component [6,7] to function both in cell-free extracts and with purified components. We have also shown previously [8] that acetylene reduction in nitrogen-fixing cultures of *R. rubrum* is inhibited by NH_4^+, glutamine and asparagine. This effect has also been shown in *Rhodopseudomonas palustris* by Zumft and Castillo [9] who called this a 'switch-off/switch-on' effect.

In this communication we report an investigation indicating a relation between the dependence on the membrane component and the 'switch-off/switch-on' effect by NH_4^+ in vivo. We have also studied hydrogen production by purified components with respect to its dependence on the membrane component and the influence of different component ratios on hydrogen production, acetylene reduction and ATP hydrolysis. We will use the nomenclature of Eady et al. [10] to designate the two nitrogenase components, referring to the MoFe protein as Rr1 and to the Fe protein as Rr2.

Materials and Methods

R. rubrum strain S1 was grown heterotrophically in the light under an atmosphere of N_2/CO_2 (95 : 5, v/v) in the synthetic medium of Bose et al. [11] with omission of the ammonium sulphate. The cells were harvested and broken, and a crude extract was produced as previously reported [6]. The nitrogenase components Rr1 and Rr2 were purified from the crude extract according to our recently published method [4].

The membrane component was solubilized from washed chromatophores as described [6]. The solution containing the membrane component was dialyzed overnight against 25 mM Tris-HCl (pH 7.8) containing 0.5 mg $Na_2S_2O_4$/ml and then applied on to a DEAE-Sepharose Cl-6B column (2.4 × 10 cm). After washing with buffer the membrane component was eluted with 0.15 M NaCl in buffer. The fractions containing the membrane component were pooled and used in the experiments. Activated Rr2 was produced by incubating purified Rr2 with the membrane component in a mixture containing 4 mM ATP, 20 mM $MgCl_2$, 10 mM creatine phosphate, 0.3 mM $MnCl_2$, 1.5 units of creatine phosphokinase/ml, 0.1 mg $Na_2S_2O_4$/ml in 50 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid (Hepes), pH 7.4, under argon 30 min at 30°C. The incubation mixture was then applied on to a DEAE-Sepharose Cl-6B column (2.4 × 3 cm). The column was washed with 30 ml of 0.25 M NaCl in 25 mM Tris-HCl (pH 7.7) and activated Rr2 was eluted with 0.5 M NaCl in the same buffer.

Acetylene reduction by nitrogenase was measured according to Burris [12] and hydrogen production by nitrogenase was determined by gas chromatography using a thermal conductivity detector [13]. Phosphate was determined

by the method of Furchgott and Gubareff [14]. Protein concentrations were determined by the method of Goa [15].

Results

'Deactivation' of nitrogenase in vivo

We have previously shown [8] that addition of NH_4^+ to a culture of nitrogen-fixing *R. rubrum* leads to a decrease in nitrogenase activity. In order to test if this 'switch-off/switch-on' effect is related to the activation by the membrane component, ammonium acetate was added to a nitrogen-fixing culture that had been nitrogen starved by gassing with argon for 14 h. 30 min after the addition of NH_4^+ the activity was 15% of the control, to which sodium acetate was added. A crude cell extract was produced as described above and used to determine the activity. The results (Table I) clearly show that the addition of NH_4^+, which is followed by a 'switch-off' in vivo produce 'deactivation' of nitrogenase. This phenomenon can be reversed by adding the membrane component to the reaction mixture.

Dependence on membrane component for hydrogen production

The nitrogenase components, Rr1 and Rr2, were purified as described previously [4] and the purity checked by SDS electrophoresis [16,17]. Rr1 ran as a single band and no other band could be detected. Rr2 produced one major band, the contaminating proteins being less than 2%. (Some preparations of Rr2 showed two bands with molecular weight 31 500 and 29 500, in agreement with those of Ludden and Burris [5].) SDS electrophoresis of activated Rr2 produced the same bands as that of 'deactivated'. Activated Rr2 was not further activated by addition of membrane component to the reaction mixture which is in agreement with the results of Ludden and Burris [7].

We have previously shown [4] that the time curve for acetylene reduction by purified Rr1 and Rr2 shows a lag which can be eliminated by preincubation of

TABLE I

ACTIVITY OF CRUDE EXTRACTS IN RELATION TO THE 'SWITCH-OFF' EFFECT

0.1 ml of solubilized membrane component (protein concentration 1.25 mg/ml) was used. The reaction was carried out in 12.5-ml injection bottles. The reaction mixture contained 4 mM ATP, 20 mM $MgCl_2$, 10 mM creatine phosphate, 1.5 units creatine phosphokinase/ml in 50 mM Hepes, pH 7.3. After gassing with argon, 0.1 ml of 20 mg $Na_2S_2O_4$/ml was added immediately prior to addition of enzyme fractions and 1.0 ml of acetylene. The total volume was 1.7 ml. The assay was run at 30°C and the reaction stopped by addition of 0.5 ml 20% $HClO_4$. 0.5 ml of the gas phase was analyzed for ethylene and 100 µl was analyzed for hydrogen, both by gas chromatography. Specific activities are based on the protein concentration in the crude extract.

	nmol C_2H_4/min per mg protein	
Addition to N_2-fixing culture:	4 mM ammonium acetate	4 mM sodium acetate
Crude extract	6.0	50.4
Crude extract + membrane component	43.0	53.4
Activation by membrane component (X)	7.2	1.1

Rr2 with the membrane component. The results depicted in Fig. 1 clearly show the same dependence on the membrane component for hydrogen production by Rr1 and 'deactivated' Rr2.

Hydrogen production with different Rr1/Rr2 ratios

Hydrogen production, both in absence and in presence of acetylene, with different molar ratios of Rr1/Rr2 with the amount of Rr2 constant was measured. (The ratios were based on the molecular weights [4] and specific activities [4,5] determined previously.) The ATP/2 e^- values were estimated by measuring the release of phosphate in presence of reductant, i.e. $Na_2S_2O_4$. The reductant-independent ATP hydrolysis was found to be less than 1% of the total. The 2 e^- values were calculated as the sum of ethylene and hydrogen produced. As can be seen in Fig. 2 acetylene reduction decreases at high Rr1/Rr2 ratios and the hydrogen production increases although the total electron flow decreases. The ATP/2 e^- value also increases at high Rr1/Rr2 ratios. Hydrogen production under argon reaches a maximum and decreases at high Rr1/Rr2 ratios (Fig. 2).

Hydrogen production at different acetylene concentrations

Hydrogen production by nitrogenase is inhibited in the presence of other

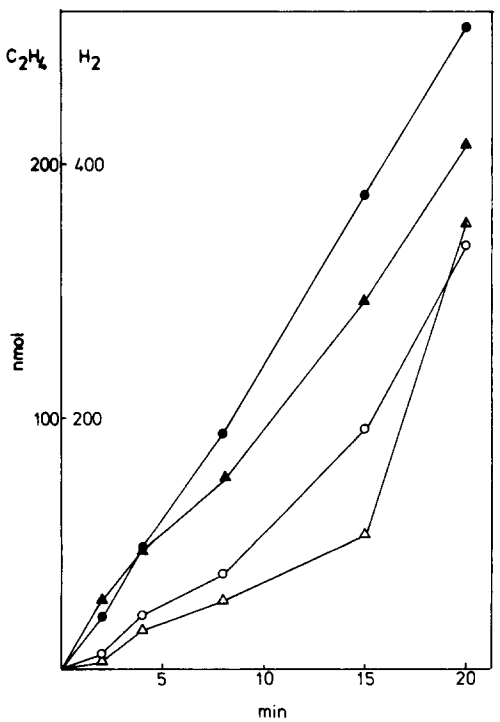

Fig. 1. Preincubation of the membrane component with purified Rr1 and Rr2. The assay was performed as described in Table I. 0.26 mg Rr1, 0.11 mg Rr2 and 1 mg of membrane component were used. △——△, H_2 production under Ar; ▲——▲, H_2 production under Ar after 6 min preincubation; ○——○, acetylene reduction, and ●——●, acetylene reduction after 6 min preincubation.

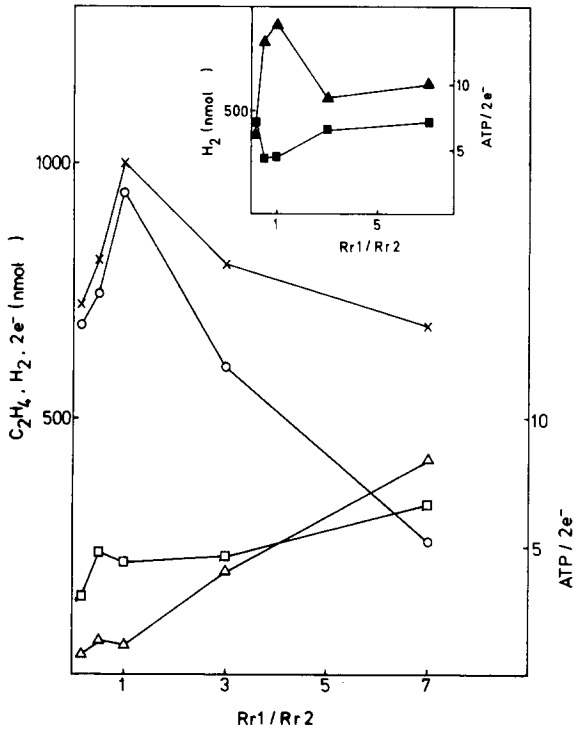

Fig. 2. Acetylene reduction, hydrogen production and ATP/2 e^- values at different Rr1/Rr2 ratios. The assay was performed as described in Table I. Reaction time was 20 min. Phosphate was determined as described in Materials and Methods. ○———○, acetylene reduction; △———△, concomitant H_2 production; X———X, total electron flow; □———□, ATP/2 e^- values. Insert shows hydrogen production under Ar at the same Rr1/Rr2 ratios. ▲———▲, H_2 production; ■———■, ATP/2 e^- values.

substrates for nitrogenase and acetylene has been reported to give the strongest inhibition [18]. Since different ratios of Rr1/Rr2 give different rates of hydrogen production we tested the inhibition of the hydrogen production by acetylene at different Rr1/Rr2 ratios. As can be seen in Fig. 3 the strongest inhibition is produced at low Rr1/Rr2 ratios, i.e. at the ratios giving the highest acetylene reduction rates. The inhibition of hydrogen production corresponds to the increase in acetylene reduction and consequently the total electron flow is constant.

Effect of CO on hydrogen production and acetylene reduction

CO is reported to inhibit all reductions catalyzed by nitrogenase except the reduction of protons [19]. The effect of CO on acetylene reduction by *R. rubrum* nitrogenase is shown in Fig. 4. Hydrogen production with acetylene present increases with increasing CO concentrations reaching the same values as hydrogen production under argon, which is unaffected by CO.

Discussion

Nitrogenase from *R. rubrum* has the unique property of being dependent on a membrane component [6,7]. The molecular properties of Rr1 and Rr2 are

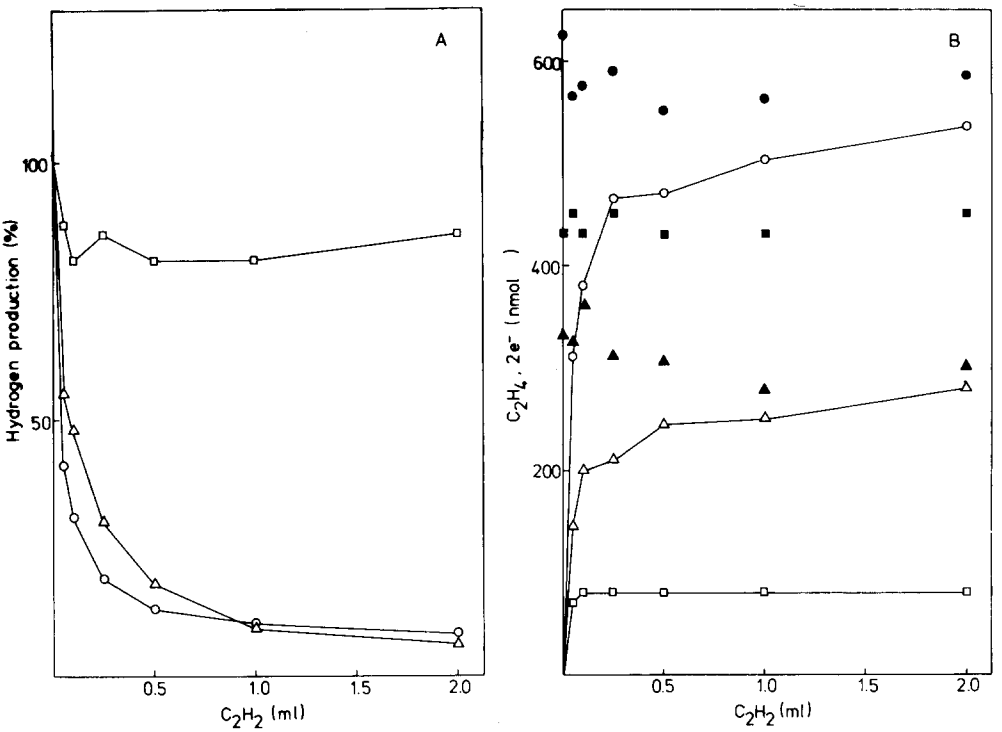

Fig. 3. Inhibition of hydrogen production by acetylene at different Rr1/Rr2 ratios. The assay was performed as described in Table I, except that different volumes of acetylene was added as shown in the figure. The reaction time was 15 min. (A) Hydrogen production activity at the following Rr1/Rr2 ratios: □——□, 7 : 1; ○——○, 1 : 1, and △——△, 1 : 7. (B) Acetylene reduction and total electron flow in the experiments in (A). Acetylene reduction at Rr1/Rr2: □——□, 7 : 1; ○——○, 1 : 1; △——△, 1 : 7. Total electron flow at Rr1/Rr2: ■, 7 : 1; ●, 1 : 1; ▲, 1 : 7.

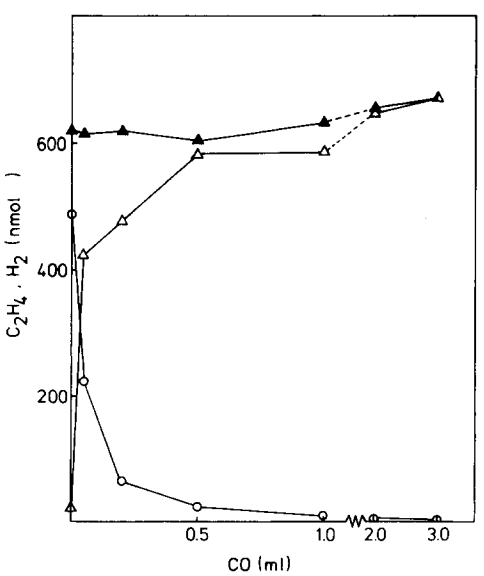

Fig. 4. Inhibition by CO. The assay was performed as described in Table I. CO was added before the addition of Rr1 (0.26 mg) and activated Rr2 (0.085 mg). Reaction time was 15 min. ●——●, actylene reduction; △——△, concomitant H_2 production; ▲——▲, H_2 production under Ar.

similar to those of nitrogenase components from other organisms [4,5] although Ludden and Burris [5] have shown that Rr2 contains phosphate, ribose and adenine which they suggest render the Rr2 inactive. Arnon and coworkers (personal communication) have shown that the nitrogenase complex from nitrogen-starved cells has a higher molecular weight than that from cells to which ammonia was added.

In this communication we have shown that the membrane component also is necessary to elicit hydrogen production by purified *R. rubrum* nitrogenase components, which confirms that Rr2 has to be activated in order to reduce Rr1. We have also shown that there is a relation between the NH_4^+ concentration in the culture and the concentration of activated Rr2. This would then indicate that the function of the activation system is to keep the Rr2 in the activated form only when the concentration of NH_4^+ or a comparable nitrogen compound, e.g. Gln, Asn, is low. The amount of reducing equivalents and ATP lost by hydrogen production would consequently be minimized. Since compounds like NH_4^+, Gln and Asn that produce the 'switch-off' effect in vivo do not have any effect in cell-free extracts [8], one may assume that some other compound, possibly in the nitrogen metabolism, is involved in this 'switch-off' effect. Further studies are clearly needed to understand this mechanism of activation and 'deactivation'. Such investigations should include characterization of the solubilized membrane component, which so far has resisted our attempts of purification.

The results from the experiments with different Rr1/Rr2 ratios confirm the results of Ljones and Burris [20] showing that the ATP/2 e^- values increase with excess of the MoFe protein over the Fe protein. At the ratio of Rr1/Rr2 giving maximal activity the ATP/2 e^- value is 4—5 (Fig. 2), which is in good agreement with reports on other nitrogenase systems [21].

The data from the experiments on acetylene reduction and hydrogen production under argon with different component ratios indicate that the functional complex consists of one Rr1 and one Rr2. Different compositions of the nitrogenase complex have been suggested for nitrogenase isolated from other organisms: *Klebsiella pneumoniae* and *Azotobacter chroococcum* 1 : 1 [22], *Clostridium pasteurianum* 1 : 2 [1] and *Corynebacterium autotrophicum* 2 : 1 for acetylene and N_2 reduction and 3 : 1 for hydrogen production [23]. Further investigations are clearly needed to understand the compositions of the functioning nitrogenase complex.

Since nitrogenase catalyzes the reduction of several substrates [18] great efforts have been made to explain the mechanism(s) of these reactions. It is generally agreed that reduction of the substrates takes place on the MoFe protein, which is reduced by the Fe protein. Hwang et al. [2] studied the inhibition of nitrogenase and proposed that there are five sites on the MoFe protein. An alternative model was suggested by Silverstein and Bulen [24] and Davis et al. [3] which propose that nitrogenase can exist in different states affected by the component ratio and the ATP level. Stiefel [25] has proposed a two metal site model which takes in account the inhibition data [2] but also includes the different states of the MoFe protein. Our data depicted in Fig. 4 show that CO interferes with the acetylene reducing site/state redirecting the electrons to the reduction of protons, which is unaffected by CO. This

indicates that the CO-binding site does not overlap with the proton-reducing site, to an extent preventing hydrogen production.

Acetylene inhibits hydrogen production but our results show that the degree of inhibition is dependent on the component ratio. Excess of Rr1 gives the smallest inhibition and at the same time the total electron flow decreases. These observations can be explained by assuming that when the MoFe protein is in excess it will be reduced at a lower rate by the Fe protein to a state at which it can reduce the substrates. Consequently, the total electron flow will decrease. The results also indicate that acetylene is less efficiently bound and/or reduced, when the MoFe protein is less reduced. Since both acetylene reduction and hydrogen production are two-electron reactions the results indicate that the state of reduction of the MoFe protein determines which substrate is bound to the site(s) of reduction. Clearly more data on the organization of the Mo and Fe in the active site is needed for a more detailed understanding of the mechanism of substrate reduction. The investigations on the Fe-Mo cofactor [26] and the Mössbauer studies [27] have already provided valuable information.

The results show that *R. rubrum* nitrogenase, when the Fe protein has been activated by the membrane component, fundamentally exhibits the same reaction characteristics as nitrogenases from other organisms. The process of activation of the Fe protein which seems to function as a regulation dependent on the concentration of some unknown nitrogen metabolite has so far been clearly demonstrated only in *R. rubrum*. There are indications that the same phenomenon occurs in *Rps. palustris* [9] and the question arises whether this is a more common property of photosynthetic organisms than has hitherto been discovered.

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